Connexin43 and Connexin45 Form Heteromeric Gap Junction Channels in Which Individual Components Determine Permeability and Regulation

Agustin D. Martinez, Volodya Hayrapetyan, Alonso P. Moreno, Eric C. Beyer

Abstract—Two gap junction proteins, connexin43 (Cx43) and connexin45 (Cx45), are coexpressed in many cardiac and other cells. Homomeric channels formed by these proteins differ in unitary conductance, permeability, and regulation. We sought to determine the ability of Cx43 and Cx45 to oligomerize with each other to form heteromeric gap junction channels and to determine the functional and regulatory properties of these heteromeric channels. HeLa cells were transfected with Cx43 or (His)$_6$-tagged Cx43 or sequentially transfected with both connexins. Immunoblots verified production of the transfected connexins, and immunofluorescence demonstrated that they were colocaled in the HeLa-Cx43(His)$_6$/Cx45 cells. Connexons were solubilized from HeLa-Cx43(His)$_6$/Cx45 cells by using Triton X-100 and were applied to a Ni$^{2+}$-NTA column, which binds the His$_6$ sequence. Cx45 was coeluted from the column with Cx43(His)$_6$, demonstrating that some hemichannels contain both connexins. Single-channel recordings showed that the HeLa-Cx43(His)$_6$/Cx45 cells exhibited single-channel conductances that were not observed in cells expressing either connexin alone. Dye-coupling experiments showed that HeLa-Cx43(Cx43(His)$_6$ cells readily passed Lucifer yellow and N-(2-aminoethyl)biotinamide hydrochloride (neurobiotin); in contrast, HeLa-Cx45 and HeLa-Cx43(His)$_6$/Cx45 cells showed extensive intercellular passage of neurobiotin but little coupling with Lucifer yellow. Treatment with the protein kinase C activator 12-O-tetradecanoylphorbol 13-acetate reduced junctional conductance in cells expressing Cx43, Cx45, or both connexins, but it reduced the extent of neurobiotin transfer only in HeLa-Cx43(His)$_6$ and HeLa-Cx43(His)$_6$/Cx45 cells but not in the HeLa-Cx43 cells. Thus, biochemical and electrophysiological evidence suggests that Cx43 and Cx45 extensively mix to form heteromeric channels; however, individual connexin components dominate aspects of the physiological behavior of these channels. (Circ Res. 2002;90:1100-1107.)

Key Words: intercellular communication | heteromeric channels | gap junctions | permeability | protein kinase C

Gap junctions contain intercellular channels that connect the cytoplasm of adjacent cells. They permit the passage of ions and small molecules between cells, providing a direct coordination of electrical and metabolic functions. In electrically excitable cells, such as cardiac myocytes, intercellular communication is critical for normal current flow. Derangements of cardiac gap junctions are associated with abnormal conduction and developmental defects. Gap junction channels are constructed of two hemichannels (connexons), each contributed by one of the opposing cells. These hemichannels are hexamers of protein subunits called connexins. Several connexins, including connexin37, connexin40 (Cx40), connexin43 (Cx43), and connexin45 (Cx45) form gap junctions between cells of the cardiovascular system.1

Expression of a single connexin by a cell is sufficient to establish gap junctional intercellular communication. The physiological properties of homomeric-homotypic channels, in which all 12 subunits are the same connexin, have been extensively characterized by use of various expression systems, and they differ for different connexins. For example, the unitary conductance of homomeric-homotypic Cx43 channels (100 to 120 pS) is greater than that of homomeric-homotypic Cx45 channels (30 to 40 pS).2 Cx43 channels are permeable to many different dye tracers, including Lucifer yellow, carboxyfluorescein, dichlorofluorescein, and N-(2-aminoethyl)biotinamide hydrochloride (neurobiotin), whereas Cx45 channels show less passage of Lucifer yellow and the fluorescein derivatives.3-5 Both Cx43 and Cx45 are phosphoproteins, and activation of kinases, such as protein kinase C (PKC), may have differential effects on junctional conductance (gj) in cells expressing these connexins.3-7

Many cells contain >1 connexin. The properties of intercellular communication between such cells should be determined by the properties of channels containing either connexin alone and channels containing a mixture of connexins. If two similarly coexpressed connexins are capable of freely

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mixing, channels containing two connexins in the same hemichannel (heteromeric channels) will be far more abundant than those formed of homomeric hemichannels (ie, those containing only one connexin). Therefore, to understand cardiac cellular coupling, it is important to understand the potential ability of two coexpressed cardiac connexins to form heteromeric channels and the properties of such channels (if they do exist).

Biochemical evidence has established the existence of heteromeric hemichannels in extracts of adult rodent or avian tissues, and of baculovirus-infected or plasmid-transfected cells. Double whole-cell patch-clamp studies of connexin-transfected cells have shown that in some cases, the heteromeric channels have properties that differ from those of channels formed of either connexin alone, whereas in other cases, the properties of heteromeric channels do not differ from those predicted from a combination of homomeric and heterotypic channels.

In the present study, we focused on the possible heteromeric interactions of Cx43 and Cx45. Both Cx43 and Cx45 are found together in a number of cells of the cardiovascular system (in atrial and ventricular myocytes, the distal conducting system, and some vascular smooth muscle cells). Cx43 and Cx45 are also found together in astrocytes, myometrial smooth muscle cells, and osteoblasts. When coexpressed, Cx43 and Cx45 colocalize to identical distributions and gap junction structures. Moreover, osteoblastic cell lines expressing both connexins might make heteromeric gap junction channels and that the formation of such mixed channels might have significant ramifications for understanding cardiac intercellular communication.

Materials and Methods

Cell Culture and Transfection
We have previously described the production of HeLa cells transfected with rat Cx43 containing a (His)6 epitope appended to its carboxyl terminus [HeLa-Cx43(His)] or transfected with chicken Cx45 (HeLa-Cx45). (Previously characterized physiological properties appear identical for chicken and mammalian Cx45.) Clones expressing both connexins [HeLa-Cx45/Cx43(His)] cells) were obtained by sequential transfection of HeLa-Cx43 cells with the pcDNA3.1-Cx43(His) construct and selection in hygromycin (1 mg/mL). Data are presented for a single clone that exhibited abundant expression of both Cx43(His) and Cx45, but consistent physiological data were obtained in more limited studies of other clones (containing differing ratios).

Immunohistochemistry and Immunoblots
A rabbit polyclonal antibody to Cx43 has been characterized previously. Mouse monoclonal antibodies to Cx43, Cx45, and the His tag were obtained from Chemicon. Single- and double-label immunofluorescence microscopy was performed essentially as described previously with the use of cells grown on glass coverslips, fixed in methanol/acetone, and permeabilized with 1% Triton X-100. Cy3-conjugated goat anti-mouse IgG and Cy2-conjugated goat anti-rabbit antibodies were obtained from Jackson ImmunoResearch. Cells were examined by using a Zeiss Axioptit II microscope equipped for epifluorescence and digital microscopy.

For immunoblots, cell cultures were rinsed with PBS, scraped into cold PBS containing 2 mmol/L phenylmethylsulfonyl fluoride, and centrifuged. After the supernatant was discarded, cell pellets were frozen in liquid nitrogen and stored at −80 °C. Cell pellets were lysed by sonication in water containing protease and phosphatase inhibitors. Immunoblot analyses were performed as described previously by using 100 μg protein or fractions eluted from a Ni2+-NTA column (Novagen).

Connexon Solubilization and Affinity Purification With Use of Ni2+-NTA Column
Connexons were solubilized by incubation of a cell lysate with 1% Triton X-100, followed by ultracentrifugation at 100 000g. Supernatants containing only one connexin. Therefore, to understand cardiac permeability properties.

Microinjection of Gap Junction Tracers
Cells cultured on coverslips (80% to 100% confluent cultures) were impaled with a micropipette filled with 150 mmol/L LiCl, 4% Lucifer yellow (charge = −2, molecular weight 457, Sigma Chemical Co), and 4% neurobiotin (charge = +1, molecular weight 3228; Vector Laboratories). Solutions were microinjected with a picosyringe (model PLI-188, Nikon Inc) by using 0.2- to 0.3-second pulses of 1 to 2 psi; cells were impaled for 0.5 to 1 minute. After the microinjection, the cells were fixed with paraformaldehyde (4% in PBS) for 30 minutes and then permeabilized with methanol/acetone (1:1) for 2 minutes at room temperature. The neurobiotin tracer was detected after staining the cells with streptavidin-Cy3 conjugate (Sigma). The extent of intercellular transfer of both tracers was determined by recording the number of adjacent cells containing the tracer after visualization by epifluorescence and digital microscopy. In some experiments, the cells were treated with 200 mmol/L 12-O-tetradecanoylphorbol 13-acetate (TPA) for 30 minutes before the microinjections.

Electrophysiology
A dual whole-cell voltage-clamp technique was applied to measure the gj between cells with the use of a CsCl pipette solution. In all experiments, the resistance of the electrodes had a maximum value of 5 MΩ, and extra suction was applied to reduce the series resistance. The initial current was measured at the beginning of the voltage pulses with the use of a low-pass filter of 5 kHz. Total junctional current measurements before and after TPA were performed in cell pairs from sister cultures plated 18 to 24 hours before study, which were well attached to the recording chamber and contained at least 5 μm of opposing membranes.

Single-channel currents were measured by using freshly split cells, where gj was low. Unitary junctional currents were recorded during long voltage steps of 10 seconds applied to one of the cells. Amplitudes of unitary opening or closing events were measured by using a digitizing board (Summagraphics with SigmaScan software, Jandel) from the chart recorder paper (Gould Windograph), where current traces were filtered at 100 to 500 Hz. Frequency distribution histograms of the events and gaussian distribution best fits were calculated for each experiment (Origin, Microcal). Each event was defined as the current transition between channel states, where the residence time in each state was >20 ms. All-points histograms were generated by using pClamp protocols from traces filtered at 200 to 500 Hz and digitized at 1 kHz. Digitized points were grouped into 128 points per bin. Multiple gaussian functions were also obtained by following the Levenberg-Marquardt algorithm to determine the best bell-shaped curves for gaussian probability distribution functions.
Immunochemical Characterization of Transfected HeLa Cells

To study the possible hetero-oligomerization of Cx43 and Cx45, we introduced Cx45 or Cx43 containing the (His)₆ sequence appended to its carboxy terminus [Cx43(His)₆] or both connexins into communication-deficient HeLa cells by stable transfection. The (His)₆ tag was added to facilitate the isolation of connexons. Immunoblotting and immunofluorescence confirmed the production of immunoreactive Cx45 and Cx43(His)₆ in the transfected HeLa cell clones (Figures 1 and 2). In immunoblots, Cx45 was detected as a single band of 48 kDa, and Cx43(His)₆ was detected as multiple bands of 42 to 46 kDa, which likely correspond to the nonphosphorylated and phosphorylated forms described for Cx43 in many systems (Figure 1). The immunoblots confirmed that the HeLa-Cx43(His)₆/Cx45 cells were producing substantial amounts of both connexins.

Immunofluorescence analysis showed that immunoreactive connexin proteins were produced and located in a distribution expected for gap junction proteins. Single-label immunofluorescent staining of HeLa-Cx43(His)₆ (Figure 2A) and of HeLa-Cx45 (Figure 2B) cells showed intense labeling for the appropriate connexin at cellular interfaces (likely representing gap junction structures) and in a perinuclear distribution within the cytoplasm (likely representing connexin within the biosynthetic pathway). Single-label immunofluorescent staining of HeLa-Cx43(His)₆/Cx45 cells showed a very similar pattern of staining (not shown). Moreover, double-label immunofluorescence staining of HeLa-Cx43(His)₆/Cx45 cells showed virtually identical and superimposable distributions (colocalization) of Cx43(His)₆ and Cx45 at plasma membrane appositions and in intracellular compartments (Figures 2C through 2G). Reaction of anti-Cx43 or anti-Cx45 antibodies with untransfected HeLa cells or HeLa cells transfected only with the other connexin gave no detectable staining (not shown).

Coelution of Cx45 With Cx43(His)₆ From Ni²⁺-NTA Affinity Column

To examine the possible hetero-oligomeric association of Cx45 with Cx43 in connexons, we used an affinity purification strategy similar to that used to show heteromeric association of Cx40 and Cx43. Triton X-100–soluble extracts containing connexons from transfected HeLa cells were applied to a Ni²⁺-NTA column, which has a high affinity for (His)₆ and, therefore, should bind connexins containing this tag (which may bring along associated proteins). Starting material and column fractions were analyzed by immunoblotting (Figure 3). We observed that Cx45 eluted with the Cx43(His)₆ when connexons from HeLa-Cx43(His)₆/Cx45 cells were affinity-purified (Figure 3, top and middle panels). In contrast, when identical procedures were applied to Triton X-100–soluble material from HeLa-Cx45 cells, all Cx45 immunoreactivity was detected in the flow-through and initial washes, suggesting that Cx45 did not bind to the Ni²⁺-NTA column (Figure 3, bottom panels). Similarly, we found no evidence of Cx45 binding to the columns when Triton extracts were prepared from HeLa-Cx43(His)₆ and HeLa-Cx45 cells that were cultured separately and the extracts were mixed before application to the column or when extracts were prepared from cocultured HeLa-Cx43(His)₆ and

Figure 1. Immunoblot analysis of connexin production by stably transfected HeLa cells. Extracts were prepared from HeLa cells transfected with Cx43(His)₆, Cx45, or Cx43(His)₆/Cx45 and immunoblotted to detect Cx43 or Cx45. Immunoreactive bands were detected only in the extracts from the appropriate transfected cells.

Figure 2. Immunofluorescence analysis of connexin production and localization in stably transfected HeLa cells. A, HeLa-Cx43(His)₆ cells reacted with anti-Cx43 antibodies. B, HeLa-Cx45 cells reacted with anti-Cx45 antibodies. C through G, Double-label immunofluorescence analysis of HeLa-Cx43(His)₆/Cx45 cells showing anti-Cx43 immunoreactivity (C and E), anti-Cx45 immunoreactivity (D and F), and superposition of both signals (G). Bar=7 μm (A through D) and 2 μm (E through G).
where $V_{o}$ (voltage at which $G_{ss}$ is half maximal) corresponds to from HeLa-Cx43(His)$_6$/Cx45 cells, but no Cx45 was detected in cally eluted from the column loaded with material were specifi
tically, and eluted with imidazole. Fractions were collected and analyzed by immunoblotting with the use of anti-Cx43 (top) or anti-Cx45 (middle and bottom) antibodies. Both Cx43 and Cx45 were successfully transfected HeLa cells. Triton X-100 solubilized material from HeLa-Cx45 cells (bottom) was applied to Ni$^{2+}$-NTA column, washed extensively, and eluted with imidazole. Fractions were collected and analyzed by immunoblotting with the use of anti-Cx43 (top) or anti-Cx45 (middle and bottom) antibodies. Both Cx43 and Cx45 were specifically eluted from the column loaded with material from HeLa-Cx43(His)$_6$/Cx45 cells, but no Cx45 was detected in eluted fractions from HeLa-Cx45 cells.

Figure 3. Affinity purification of solubilized connexons from stably transfected HeLa cells. Triton X-100 solubilized material from HeLa-Cx43(His)$_6$/Cx45 cells (top and middle) or from HeLa-Cx45 cells (bottom) was applied to Ni$^{2+}$-NTA column, washed extensively, and eluted with imidazole. Fractions were collected and analyzed by immunoblotting with the use of anti-Cx43 (top) or anti-Cx45 (middle and bottom) antibodies. Both Cx43 and Cx45 were successfully transfected HeLa cells. Triton X-100 solubilized material from HeLa-Cx45 cells (bottom) was applied to Ni$^{2+}$-NTA column, washed extensively, and eluted with imidazole. Fractions were collected and analyzed by immunoblotting with the use of anti-Cx43 (top) or anti-Cx45 (middle and bottom) antibodies. Both Cx43 and Cx45 were specifically eluted from the column loaded with material from HeLa-Cx43(His)$_6$/Cx45 cells, but no Cx45 was detected in eluted fractions from HeLa-Cx45 cells.

HeLa-Cx45 cells (not shown). These data demonstrate that Cx45 association with Cx43 required coexpression in the same cell and did not occur after detergent solubilization or heterotypic interactions. The specific binding of Cx45 to Ni$^{2+}$-NTA when connexons were solubilized from HeLa-Cx43(His)$_6$/Cx45 cells suggested oligomeric interactions to form heteromeric connexons.

**Unitary Conductances of Gap Junction Channels in Transfected HeLa Cells**

The double whole-cell patch-clamp approach was used to obtain single-channel recordings from low-conductance pairs of HeLa cells transfected with Cx43(His)$_6$ or Cx45 or sequentially transfected with Cx43(His)$_6$ and Cx45. The maximal single-channel conductance was determined by using low transjunctional voltage protocols (<60 mV) in cells that had been plated for 1 to 4 hours. Examples of representative recordings from HeLa-Cx43(His)$_6$ and HeLa Cx43(His)$_6$/Cx45 cells are shown in Figures 4 and 5, and event histograms summarizing the channel data obtained from all transfectants are presented in Figure 6.

The behavior of Cx43(His)$_6$ (Figure 4) was rather similar to that of wild-type Cx43. Single-channel recordings showed the presence of a main state of $\approx 120$ pS and a residual state of 30 pS (Figures 4A and Figure 6, top left panel). This value did not differ significantly from previous measurements of the unitary conductance of wild-type Cx43 as expressed in similar culture systems. Even when analyzed in multichannel recordings, small events (50 to 100 pS) were rare (<5% of events). The Cx43(His)$_6$ channels did exhibit less voltage dependence than did the wild-type Cx43. They had remarkably high activity despite voltage pulses as great as 100 mV (Figure 4A). The voltage dependence was examined at various transjunctional voltages, and the corresponding series of inactivating currents is shown in Figure 4B. At 60 mV, the current did not inactivate at all. A comparison of the plots of normalized steady-state conductance versus voltage (Figure 4C) also suggests a somewhat lower voltage sensitivity for Cx43(His)$_6$, than for wild-type Cx43.

The unitary conductance of the gap junction channels ($g_j$) in HeLa-Cx45 cells was also obtained: it was best fit by a single gaussian function at $g_j = 38 \pm 14$ (5 experiments, 1045 events) (Figure 6, top middle panel). This value was indistinguishable from that obtained in previous studies of Cx45 in similar expression systems.

Single-channel events of many different sizes were observed in pairs of cells coexpressing Cx43(His)$_6$, and Cx45 (Figure 5). Many of these channel events did not correspond to channel sizes detected in the HeLa-Cx43(His)$_6$, or HeLa-Cx45 cell pairs. These data also differed dramatically from our previous analyses of heterotypic Cx43-Cx45 channels, which had a single unitary conductance of 60 pS. Figure 5 contains a representative trace for which multiple different unitary current transitions were recorded from a pair of HeLa-Cx43(His)$_6$/Cx45 cells during a 60-mV voltage pulse. Multiple transitions were evident, and detailed analysis of this recording demonstrates the multiple different unitary conductances (Figures 5a through 5g). An event histogram of the channel data from HeLa-Cx43(His)$_6$/Cx45 cell pairs (Figure 6, top right panel) required $\geq 8$ gaussian functions for best fitting.

**Figure 4.** Properties of gap junction channels recorded from pairs of HeLa-Cx43(His)$_6$ cells. A, Junctional current trace showing unitary current events detected during a 100-mV transjunctional voltage pulse. The all-points histograms at the right indicate that the main transitions correspond to channels with a conductance of 118 to 120 pS. B, Junctional current traces obtained during a protocol of pulses from $-100$ to 100 mV. C, Plot of normalized steady-state junctional conductance ($G_{ss}$) vs transjunctional voltage ($V_j$) for pairs of cells expressing wild-type Cx43 (●, n=6) or Cx43(His)$_6$ (○, n=4). The smooth curves represent the best fit to a Boltzmann equation, where $V_c$ (voltage at which $G_{ss}$ is half maximal) corresponds to $\pm 60$ mV for Cx43 and $\pm 74$ mV for Cx43(His)$_6$.0

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![Image](http://circres.ahajournals.org/Download/file/10578/65260/5078886/figure3_small.jpg)

![Image](http://circres.ahajournals.org/Download/file/10578/65260/5078886/figure2_small.jpg)
control conditions, the extent of neurobiotin transfer was similar among all of the HeLa transfectants (Figure 7, hatched bars); the number of dye-filled cells was 21.5 ± 3.2 for HeLa-Cx43(His)_6 cells, 15 ± 2.5 for HeLa-Cx45 cells, and 22.9 ± 3.4 for HeLa-Cx43(His)_6/Cx45 cells. In contrast, extensive transfer of Lucifer yellow (Figure 7, solid bars) was observed only for HeLa-Cx43(His)_6 cells (11.2 ± 2.9 dye-filled cells), whereas the HeLa-Cx45 and HeLa-Cx43(His)_6/Cx45 cells showed only limited transfer of this dye (0.8 ± 0.2 and 1.5 ± 0.3 dye-filled cells, respectively). Thus, the permeability properties (for these two dyes) of the HeLa-Cx43(His)_6/Cx45 cells were similar to those of cells expressing only Cx45.

Effects of PKC Activation on Intercellular Communication Between Transfected HeLa Cells

In many cell types, gap junctional communication is regulated by protein kinases. One of the most studied effects is the alteration of cellular coupling after activation of PKC by treatment of cells with the phorbol ester TPA. We hypothesized that TPA treatment might have different effects on cells expressing different connexins or making heteromeric channels.

We incubated monolayers of transfected HeLa cells with 200 nmoL/L TPA for 30 minutes before determining the extent of transfer of microinjected neurobiotin. In the HeLa-Cx43(His)_6 cells, TPA treatment led to a dramatic reduction of dye coupling (Figure 7; compare open and hatched bars). Many investigators have previously observed reductions in dye coupling after TPA treatment of cells expressing wild-type Cx43.25–27 Neurobiotin dye coupling was also dramatically reduced in HeLa-Cx43(His)_6/Cx45 cells after TPA treatment. However, in the HeLa-Cx45 cells, TPA treatment had no detectable effect on intercellular transfer of neurobiotin (Figure 7). Thus, the response of the coexpressing cells to PKC activation (reduced dye transfer) was similar to that of cells expressing Cx43 alone. Immunoblot analysis of HeLa-Cx43(His)_6 and HeLa-Cx43(His)_6/Cx45 showed an increase in the abundance of the slower mobility Cx43 forms (phospho forms) after TPA treatment but showed no effects on Cx45 (data not shown).

To determine the effect of TPA on total junctional communication, we calculated the g_j of multiple cell pairs expressing only Cx43(His)_6 or Cx45 or coexpressing both connexins. The total g_j decreased in all cell types analyzed after the application of TPA (Figure 8). For pairs of HeLa-Cx43(His)_6 cells, the total conductance was only 15% of control values after application of either concentration of TPA, whereas in HeLa-Cx45 or HeLa-Cx43(His)_6/Cx45 cell pairs, TPA treatment reduced conductance to ~30% of control values. To determine the influence of the series resistance on our measurements, we plotted all g_j data against the sum of the resistance of the recording electrodes (data not shown). Extrapolation to zero resistance indicated that even in the worst scenario, we have underestimated the TPA-induced reduction of g_j by <20%.

We also studied the effects of TPA treatment on the distribution of single gap junction channel conductance sizes (Figure 6, bottom panels). The event histograms appeared identical for HeLa-Cx45 cell pairs whether they were determined under...
control conditions or after TPA treatment. However, TPA treatment was accompanied by a reduction in the frequency of larger sized events (\(90\,\text{pS}\)) for both HeLa-Cx43(His)\(_6\) and HeLa-Cx43(His)\(_6\)/Cx45 cells (Figure 6).

**Discussion**

The data in the present study provide strong biochemical and electrophysiological evidence indicating that coexpressed Cx43 and Cx45 form heteromeric channels. When detergent

![Figures 6 and 7](https://example.com/figures6and7.png)

**Figure 6.** Event histograms for single channels detected in pairs of HeLa-Cx43(His)\(_6\), HeLa-Cx45, and HeLa-Cx43(His)\(_6\)/Cx45 cells under control conditions and after treatment with 200 nmol/L TPA. Each event histogram represents 600 events from 5 separate experiments. Transjunctional voltage (V) was \(40\,\text{mV}\). The smooth curves represent the best gaussian fits to the data. Under control conditions, the HeLa-Cx43(His)\(_6\) data were best fit by a major peak at 105 pS (with smaller peaks at 36, 73, and 126 pS); the HeLa-Cx45 data were best fit by a single gaussian function at \(V=38\pm14\); and the HeLa-Cx43(His)\(_6\)/Cx45 data required multiple gaussian functions to fit the data. The data obtained from cell pairs after TPA treatment were fitted with gaussian functions at the same conductances. The HeLa-Cx45 data showed no change. However, after TPA treatment, the unitary conductance values observed in the HeLa-Cx43(His)\(_6\) cells and in the HeLa-Cx43(His)\(_6\)/Cx45 cells showed a loss of the larger-sized events.

**Figure 7.** Intercellular transfer of microinjected dyes in transfected HeLa cells. The left panels show color photomicrographs after simultaneous injections of Lucifer yellow (A, C, and E) and neurobiotin (B, D, and F) into a single cell within a monolayer of HeLa-Cx43(His)\(_6\) (top), HeLa-Cx45 (middle), or HeLa-Cx43(His)\(_6\)/Cx45 cells (bottom) under control conditions. The bar graphs at the right show the quantification of \(\sim50\) such injections from at least 4 separate experiments. Lucifer yellow (solid bars) transferred extensively only between HeLa-Cx43(His)\(_6\) cells; the number of coupled cells was significantly greater than that observed for HeLa-Cx45 or HeLa-Cx43(His)\(_6\)/Cx45 cells. In contrast, neurobiotin (hatched bars) transfer was extensive in all of the transfected cells. After treatment with 200 nmol/L TPA (open bars), the extent of transfer of neurobiotin was substantially reduced in the HeLa-Cx43(His)\(_6\) and HeLa-Cx43(His)\(_6\)/Cx45 cells but was not significantly changed in the HeLa-Cx45 cells.
extracts containing solubilized connexons from HELa-Cx43(His)6/Cx45 cells were subjected to affinity purification, Cx45 was copurified with Cx43(His)6, even though only the Cx43 contained the tag that would bind to the column; copurification required association of Cx45 with Cx43 (in heteromers). Control experiments excluded artifactual association. Moreover, analysis of single gap junction channel events in pairs of HELa-Cx43(His)6/Cx45 cells yielded a large variety of events of sizes that were not seen in cells containing only homomeric connexons. In this respect, our data differ from those of Valiunas et al., who concluded that all of the voltage gating and most (if not all) of the single-channel events in cells coexpressing Cx43 and Cx40 could be explained by the properties of homomeric/homotypic and homomeric/heterotypic channels.

Our biochemical studies were facilitated by the incorporation of the (His)6 tag on the carboxyl terminus of Cx43. This strategy was accompanied by a theoretical risk of altering the behavior of Cx43. However, many of the functional properties of Cx43 were not altered by this modification. The unitary channel conductances observed between pairs of HELa-Cx43(His)6 cells were indistinguishable from those previously observed in cells expressing wild-type Cx43. This suggests that the diffusion of neurobiotin was not significantly affected by the reduction in g0 or that the permeability to neurobiotin of Cx45 channels increased during a simultaneous reduction of the open probability of the channels.

Previous studies have suggested that gap junction channels formed of different connexins differ in their permeability and selectivity. Cx43 channels have been shown to allow passage of many different tracer dyes, including Lucifer yellow, carboxyfluorescein, and neurobiotin; Cx45 channels are more selective, showing extensive coupling with neurobiotin but only limited coupling with Lucifer yellow or carboxyfluorescein.1,2,21 Our data from HELa-Cx43(His)6 cells and HELa-Cx45 cells are consistent with those observations. We further found that whereas neurobiotin readily crossed between HeLa-Cx43(His)6/Cx45 cells, these cells showed little intercellular transfer of Lucifer yellow. These results suggest that heteromeric channels formed by Cx43/Cx45 have permeability characteristics similar to those of homomeric Cx43 channels. Remaining studies have suggested that homomeric Cx45 channels are more selective for cations compared with anions, whereas homomeric Cx43 channels show little preference.2,28 Because the two tracers that we used (Lucifer yellow and neurobiotin) differ both in molecular weight and in charge, we cannot say whether the observed difference in transfer in HELa-Cx43(His)6/Cx45 cells is due to charge or size selectivity. Our current data may help to explain the observations of Koval et al., who noted that introduction of Cx45 into ROS cells, which endogenously express Cx43, led to decreased dye coupling when assayed with Lucifer yellow.

Cx43 and Cx45 are phosphoproteins, and their phosphorylation is increased after PKC activation.3,25,30,31 In many (but not all) Cx43-expressing cells, activation of PKC by phorbol esters is accompanied by a reduction in gap junctional communication.25,26,27 TPA-induced effects on communication via Cx45 channels have been less extensively studied; TPA treatment has been reported to lead to an acute (<30-minute) increase in total g0, whereas longer treatment (10 to 45 minutes) was associated with the appearance of smaller (~16-pS) channel events.

We found that TPA treatment led to decreased dye coupling (neurobiotin) between HELa-Cx43(His)6 cells. Similar observations have been made in a number of cell systems expressing Cx43. In contrast, TPA treatment had no effect on dye transfer between HELa-Cx45 cells. Neurobiotin coupling was also drastically reduced in HELa-Cx43(His)6/Cx45 cells after TPA treatment, suggesting that the heteromeric channels are affected by PKC-activated pathways. These results suggest that heteromeric channels formed by Cx43/Cx45 may be regulated by PKC activation in a manner similar to that in homomeric Cx43 channels.

Total g0 was reduced after TPA treatment in all of the transfected cells studied. This decrease was accompanied by a shift in unitary conductance (loss of the largest channels) for the HELa-Cx43(His)6 and HELa-Cx43(His)6/Cx45 cells. These observations are consistent with recent studies suggesting that phosphorylation of Ser368 in Cx43 is associated with decreased unitary channel conductance. The mechanism of decreased conductance in the HELa-Cx43 cells may not be the same, because unitary conductance was not changed in these cells.

In the HELa-Cx43(His)6, and HELa-Cx43(His)6/Cx45 cells, g0 and neurobiotin transfer decreased in parallel with TPA treatment. The shift to lower single-channel conductances suggests that the reduced channel size was concomitant with a reduction in the permeability to neurobiotin. Thus, the changes in permeability to neurobiotin of Cx45 and Cx43(His)6 may be guided by the presence of Cx43.

In the HELa-Cx45 cells, TPA treatment led to a substantial reduction in g0, without a change in neurobiotin permeability. This suggests either that the diffusion of neurobiotin was not significantly affected by the reduction in g0 or that the permeability to neurobiotin of Cx45 channels increased during a simultaneous reduction of the open probability of the channels.

Regardless, as previously reported by Veenstra et al., the
permeability and conductance properties of gap junction channels are not always directly correlated.

In summary, we have shown that coexpressed Cx43 and Cx45 can form heteromeric channels. Some characteristics of the heteromeric channels (Lucifer yellow permeability and TPA-induced reduction of neurobiotin transfer) are dominated by one of the connexin components. These properties appear to follow those of the most restrictive component.

These findings may have significant implications for the regulation of intercellular communication in the heart. Recent studies of freshly isolated myocardial and vascular cells have shown the presence of multiple single-channel conductances in their gap junctions consistent with the formation of heteromeric channels. Differences in the relative expression of Cx43 and Cx45 might lead to changes in the abundances of heteromeric Cx43/Cx45 gap junction channels. Such changes might be accompanied by alterations in conduction and in the intercellular permeability/flux of signaling molecules.

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References


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